EXHIBIT O

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Cancer Epidemiology, Biomarkers & Prevention

Null Results in Brief

Nitrosamines and Heme Iron and Risk of Prostate Cancer in the European Prospective Investigation into Cancer and Nutrition

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Abstract

Background: The evidence about nitrosamines and heme iron intake and cancer risk is limited, despite the biologic plausibility of the hypothesis that these factors might increase cancer risk. We investigated the association between dietary nitrosamines and heme iron and the risk of prostate cancer among participants of European Prospective Investigation into Cancer and Nutrition (EPIC).

Methods: Data on food consumption and complete follow-up for cancer occurrence was available for 139,005 men, recruited in 8 European countries. Estimates of HRs were obtained by proportional hazard models, stratified by age at recruitment, and study center, and adjusted for total energy intake, smoking status, marital status, dairy products, educational level, and body mass index.

Results: After a mean follow-up of 10 years, 4,606 participants were diagnosed with first incident prostate cancer. There was no overall association between prostate cancer risk and nitrosamines exposure (preformed and endogenous) or heme iron intake (HR for a doubling of intake: 1.00; 95% CI: 0.98–1.03 for *N*-Nitrosodimethlyamine, 0.95; 95% CI: 0.88–1.03 for endogenous Nitrosocompounds, and 1.00; 95 CI: 0.97–1.03 for heme iron).

Conclusions and Impact: Our findings do not support an effect of nitrosamines (endogenous and exogenous) and heme iron intake on prostate cancer risk. *Cancer Epidemiol Biomarkers Prev; 21(3); 547–51. ©2012 AACR.*

Introduction

Prostate cancer is the second most common cancer diagnosed in men, and it is the sixth most common cause of cancer mortality worldwide (1). It has been suggested that some aspect of a Western diet, such as a high meat intake, may increase prostate cancer risk (2, 3). There are various mechanisms by which meat could increase risk, one of which is via heme iron, which can

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Table 1. Baseline characteristics of the cohort according to quintiles of nitrosamines and heme iron intake

		Heme Iron (mg/d)		NDMA (μg/d)		ENOC (μg/d)	
		Q1	Q5	Q1	Q5	Q1	Q5
Mean intake		0.41	2.74	0.045	0.87	54.27	142.85
Age at recruitment (y)		50.44	51.37	53.33	52.26	50.99	51.35
Smoking status (%)	Never	25.99	16.93	23.21	15.07	24.5	18.98
	Former	20.33	18.02	19.64	21.67	20.78	18.21
	Smoker	12.25	26.32	15.7	24.21	13.17	23.93
	Unknown	31.13	11.44	42.86	5.79	35.51	8.89
Educational level (%)	None	7.07	31.37	33.87	9.22	18.06	16.21
	Primary school	13.05	23.94	20.74	22.18	13.62	22.91
	Technical/professional	18.77	19.99	18.36	21.76	17.58	21.19
	Secondary school	21.53	17.87	19.86	13.2	20.14	19.72
	Longer education	26.17	16.79	16.53	21.98	26	18.12
	Not specified	56.17	3.25	41.49	9.7	53.23	3.47
BMI (%)	Normal	27.57	15.55	21.49	17.29	26.01	18.35
	Overweight	17.14	21	19.16	20.85	17.53	20.12
	Obese	11.03	27.47	19.1	23.8	13.5	23.61
Marital status (%)	Single	36.13	11.99	23.24	17.79	33.37	15.6
	Married/living together	25.38	13.11	24.77	20.87	24.95	16.03
	Divorced/separated	30.32	12.74	17.67	31.34	26.95	18
	Widowed	32.15	10.99	31.37	22.29	31.63	14.29
	Unknown	3.88	36.36	10.16	17.15	5.88	29.07
Alcohol (g/d)		13.55	27.55	9.55	36.86	14.49	24.85
Red and processed meats (g/d)		31.08	117.37	53.98	133.39	28.27	158.63
Protein from dairy (g/d)		18.73	19.9	16.08	18.02	18.27	20.63
Total energy intake (Kcal/d)		2,025.73	2,818.44	2,053	2,639.4	2,038.06	2,782.98

promote endogenous production of NOCs (Nitroso-compounds; ref. 4); in addition, any sources of iron can also catalyze free radical formation leading to oxidative cell damage (5). Although a recent meta-analysis on cohort studies is not supportive of a positive association with red or processed meat (6), it does not preclude an association with certain aspects of meat intake, such as its content of heme iron, or nitrosamines. The aim of this study was to examine the association between dietary nitrosamines (endogenous and exogenous), heme iron intake and prostate cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC).

Material and Methods

The rationale and design of EPIC conducted in 23 centers in 10 European countries were described in detail elsewhere (7). A questionnaire collected information about sociodemographic and lifestyle characteristics and medical history. Dietary data were collected by validated country-specific questionnaires that recorded the usual dietary intake over the previous 12 months (7), and which were used to estimate preformed NDMA (*N*-Nitrosodimethylamine) from different food groups, predicted ENOC (endogenous NOCs; μg/d; ref. 8) and heme iron intake, as described elsewhere (9, 10).

Men were not eligible for this analysis if they had no dietary or nondietary data were missing (5,761 men). Individuals were also excluded if they were in the top or bottom 1% of the distribution of the ratio of reported energy intake to energy requirement (79 cases), to reduce the impact of implausible extreme values in the analysis (11). One male noncase from Athens, who was aged <30 at recruitment, was excluded. We also excluded subjects with an extreme heme iron intake (heme iron >4 mg/d; 2% of cases and noncases). Following these exclusions, complete data on diet and follow-up for cancer were available for 139,005 men of the 148,016 men in the original data set.

Prostate cancer case ascertainment was via cancer registries, active follow-up, and other methods as previously described (12). Proportional hazards models were used to estimate the association between nitrosamines (NDMA and ENOC) and heme iron and prostate cancer risk. Age was used as the underlying time scale, with entry time defined as age at recruitment and exit time defined as age at first prostate tumor (cases) and age at death or last complete follow-up (noncases). All models were stratified by age and center and adjusted for confounding variables (see Table 2). Subgroup analyses were also carried out according to stage and grade of the tumor, as previously described (12). Trend tests

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Table 2. Adjusted HRs for full cohort and high/low risk of prostate cancer by quartiles of heme iron, ENOC, NDMA, and meat

	Heme iron (mg/Kcal)[a]			ENOC (μg/kcal)			NDMA (μg/kcal)[b]		
	Noncases	Cases	HR	Noncases	Cases	HR	Noncases	Cases	HR
Full cohort (139,005)									
Q1	26,862	923	1.00	27,065	736	1.00	26,889	795	1.00
Q2	26,742	1,043	0.98 (0.89–1.08)	26,877	924	1.04 (0.94–1.15)	26,880	804	1.07 (0.96–1.19)
Q3	26,828	958	0.98 (0.89–1.08)	62,857	944	0.99 (0.89–1.10)	26,774	910	1.05 (0.94–1.17)
Q4	26,964	821	0.88 (0.79–0.98)	26,801	1,000	1.00 (0.89–1.11)	26,673	1,011	1.05 (0.94–1.18)
Q5	26,928	857	0.91 (0.82–1.02)	26,799	1,002	0.91 (0.81–1.03)	26,607	1,077	1.04 (0.92–1.18)
P for trend			0.03			0.04			0.95
log_2	134,324	4,602	1.00 (0.97–1.03)	170,399	4,606	0.95 (0.88–1.03)	133,823	4,597	1.00 (0.98–1.03)
Stage[c]									
Advanced									
Q1	27,603	182	1.00	27,658	143	1.00	27,523	161	1.00
Q2	27,622	163	0.77 (0.61–0.96)	27,631	170	0.97 (0.77–1.23)	27,537	147	1.00 (0.78–1.27)
Q3	27,598	188	0.84 (0.67–1.05)	27,613	188	0.93 (0.73–1.18)	27,492	192	0.98 (0.76–1.26)
Q4	27,606	179	0.77 (0.61–0.98)	27,615	186	0.84 (0.65–1.08)	27,475	209	0.93 (0.72–1.21)
Q5	27,585	200	0.87 (0.69–1.11)	27,574	227	0.97 (0.75–1.27)	27,480	204	0.93 (0.72–1.21)
P for trend			0.55			0.83			0.54
log_2	138,014	912	0.96 (0.90–1.03)	138,091	914	0.96 (0.78–1.15)	137,507	913	1.00 (0.94–1.06)
Localized									
Q1	27,503	282	1.00	27,552	249	1.00	27,398	286	1.00
Q2	27,439	346	1.01 (0.86–1.19)	27,517	284	0.86 (0.72–1.02)	27,383	301	1.20 (1.01–1.43)
Q3	27,427	359	1.07 (0.90–1.26)	27,442	359	0.98 (0.82–1.16)	27,373	311	1.12 (0.93–1.35)
Q4	27,470	315	0.91 (0.76–1.08)	27,402	399	0.99 (0.83–1.19)	27,347	337	1.15 (0.94–1.40)
Q5	27,407	378	1.01 (0.84–1.20)	27,411	390	0.85 (0.70–1.03)	27,242	442	1.23 (0.99–1.53)
P for trend			0.75			0.24			0.19
log_2	137,246	1,680	1.00 (0.94–1.06)	137,324	1,681	0.93 (0.82–1.06)	136,743	1,677	1.04 (0.98–1.09)
Uncertain									
Q1	27,326	459	1.00	27,457	344	1.00	27,336	348	1.00
Q2	27,251	534	1.05(0.92–1.20)	27,331	470	1.23 (1.06–1.42)	27,328	356	1.10 (0.86–1.18)
Q3	27,375	411	0.99 (0.85–1.14)	27,404	397	1.01 (0.86–1.18)	27,277	407	1.02 (0.87–1.20)
Q4	27,458	327	0.92 (0.79–1.07)	27,386	415	1.07 (0.91–1.26)	27,219	465	1.03 (0.87–1.21)
Q5	27,506	279	0.83 (0.71–0.99)	27,416	385	0.93 (0.78–1.10)	27,253	431	0.96 (0.80–1.15)
P for trend			0.01			0.06			0.43
log_2	136,916	2,010	1.01 (0.97–1.05)	136,994	2,011	0.96 (0.86–1.08)	136,413	2007	0.98 (0.95–1.02)
Grade[d]									
Low									
Q1	27,401	384	1.00	27,487	314	1.00	27,644	314	1.00
Q2	27,341	444	1.00 (0.86–1.15)	27,415	386	1.04 (0.89–1.21)	27,632	366	1.12 (0.96–1.31)
Q3	27,387	399	0.99 (0.85–1.15)	27,408	393	1.01 (0.86–1.18)	27,620	331	1.02 (0.86–1.20)
Q4	27,461	324	0.86 (0.73–1.01)	27,401	400	0.98 (0.82–1.15)	27,633	371	1.08 (0.91–1.29)
Q5	27,420	365	0.91 (0.77–1.07)	27,378	423	0.87 (0.73–1.04)	27,626	533	1.13 (0.93–1.36)
P for trend			0.08			0.04			0.33
log_2	137,010	1,916	1.01 (0.96–1.05)	137,089	1,916	0.92 (0.82–1.04)	138,155	1915	1.02 (0.98–1.07)
High									
Q1	27,729	56	1.00	27,752	49	1.00	27,644	40	1.00
Q2	27,731	54	0.95 (0.64–1.41)	27,747	54	1.04 (0.70–1.57)	27,632	52	1.32 (0.86–2.03)
Q3	27,739	47	0.91 (0.60–1.39)	27,762	39	0.73 (0.46–1.15)	27,620	64	1.50 (0.97–2.32)
Q4	27,733	52	1.01 (0.66–1.55)	27,740	61	1.12 (0.72–1.73)	27,633	51	1.18 (0.74–1.88)

(Continued on the following page)

Table 2. Adjusted HRs for full cohort and high/low risk of prostate cancer by quartiles of heme iron, ENOC, NDMA, and meat (Cont'd)

	Heme iron (mg/Kcal)[a]			ENOC (μg/kcal)			NDMA (μg/kcal)[b]		
	Noncases	Cases	HR	Noncases	Cases	HR	Noncases	Cases	HR
Q5	27,729	56	1.05 (0.68–1.64)	27,739	62	1.03 (0.65–1.66)	27,626	58	1.34 (0.81–2.24)
P for trend			0.68			0.71			0.68
log_2	138,661	265	0.99 (0.89–1.10)	138,740	265	1.01 (0.73–1.38)	138,155	265	1.09 (0.97–1.21)
Uncertain									
Q1	27,326	483	1.00	27,428	373	1.00	27,246	441	1.00
Q2	27,251	545	0.97 (0.85–1.10)	27,317	484	1.05 (0.91–1.21)	27,298	386	0.99 (0.86–1.15)
Q3	27,375	512	0.98 (0.86–1.12)	27,289	512	1.00 (0.87–1.16)	27,169	515	1.01 (0.86–1.18)
Q4	27,458	445	0.88 (0.76–1.02)	27,262	539	1.00 (0.86–1.16)	27,095	589	0.99 (0.84–1.16)
Q5	27,506	436	0.90 (0.78–1.05)	27,284	517	0.94 (0.80–1.10)	27,198	486	0.92 (0.76–1.10)
P for trend			0.10			0.23			0.18
log_2	136,916	2,421	0.99 (0.95–1.03)	136,580	2,425	0.97 (0.86–1.08)	136,006	2,417	0.98 (0.95–1.02)

NOTE: All models were stratified by age, and center and adjusted for educational level (none, primary school, technical/professional school, secondary school, longer education and unknown), marital status, BMI (as continuous variable), protein from dairy (continuous), smoking status (never, former, and current), lifetime intensity of smoking (for current smokers, <20 cigarettes per day and >20) and time since quitting (for former smokers, <10 years and >10 years) and total energy intake (continuous per 2,000 kcal).

[a]79 noncases missing.

[b]cases and 576 noncases missing.

[c]Prostate cancer stage was based on the TNM code: Advanced (T3 or T4 or N1+ or M1, or stage coded in the recruitment center as metastatic), localized (TNM staging score of T0/T1/T2 and N0/NX and M0 or stage coded in the recruitment center as localized) or unknown.

[d]Prostate cancer grade was defined as follow: low grade was defined as (Gleason sum <8 or equivalent) and high-grade disease (Gleason >8 or equal) or unknown.

2,592 cases (61%) had information on stage and 2,281 cases (60%) had information on grade.

Linear trends were tested by using the median value of each category as an ordinal variable.

Homogeneity of risk by stage and grade were tested by Wald test (all *P* values >0.05).

were done by assigning the median value for each quintile category and modeling this as a continuous variable. The Statistical Analysis System version 9 (SAS Institute) was used for analyses.

Results

After a mean follow-up of 11 years, 4,606 prostate cancer cases were identified. The association of lifestyle characteristics by dietary intake of nitrosamine and heme iron is shown in Table 1. In multivariable models, prostate cancer risk was not associated with intake of nitrosamines (HR for the highest vs. lowest quintile: 0.91; 95% CI: 0.81–1.03 for ENOC, and 1.04; 95% CI: 0.95–1.18 for NDMA) or heme iron (HR for the highest vs. lowest quintile: 1.00; 95 CI: 0.88–1.39). This lack of association was supported by analyses of continuous variables (Table 2). Furthermore, similar findings were found when stratifying prostate cancer cases by grade and stage.

Discussion

To our knowledge this is the first European prospective study of nitrosamines, heme iron intake, and prostate cancer risk. We found no overall association between intake of nitrosamines and heme iron and prostate cancer risk. One study reported that heme iron intake was associated with a 9% increased risk for total prostate cancer and 28% for advanced disease (3), but we did not find any associations of the dietary factors with risk regardless of stage or grade. One possible explanation for our null findings is that red meat intake (the most important source of heme iron) is also a source of selenium and zinc. The WCRF report (1) judges foods containing selenium as probably protective against prostate cancer. However, a previous analysis from our group did not find any association between plasma selenium and prostate cancer risk (13). The strengths of this study include its large size, prospective design, and adjustment of potential confounding variables.

In summary, our findings do not support the hypothesis that dietary nitrosamines and heme iron intakes are associated with the risk of developing prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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